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IMMUNIZATION OF MICE WITH PLASMID DNA CODING FOR NcGRA7 OR NcsHSP33 CONFERS PARTIAL PROTECTION AGAINST VERTICAL TRANSMISSION OF *NEOSPORA CANINUM*

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ABSTRACT: The purpose of the present study was to use direct plasmid deoxyribonucleic acid (DNA) injection to identify specific antigens that confer protection against congenital transfer of *Neospora caninum*. Inbred BALB/c mice were vaccinated before pregnancy with a recombinant plasmid containing sequences encoding *N. caninum* antigen NcGRA7 or NcsHSP33. The mice were challenged with *N. caninum* tachyzoites at 10–12 days of gestation. Whereas 100% of pups born from dams immunized with control plasmid contained detectable levels of *N. caninum* DNA in a *Neospora*-specific polymerase chain reaction assay, only 46% of pups from pCMVi-NcGRA7–immunized mice and 53% of pCMVi-NcsHSP33–immunized mice were *N. caninum* positive, and none of the mice immunized with tachyzoite extract contained *N. caninum* DNA. Thus, immunization of mice with plasmid DNA expressing *N. caninum* antigens conferred partial protection against congenital neosporosis.

Neospora caninum is a tissue cyst–forming coccidian that is an important cause of abortion in dairy cattle worldwide (Dubey, 1999). Infection of the fetus by tachyzoites is thought to arise from a reactivated tissue cyst rupture or from the ingestion of *N. caninum* oocysts. A number of research groups are seeking to develop control measures, such as vaccination, against bovine neosporosis. Studies in mice have shown that protection against neosporosis is mediated by a type I immune response involving interleukin-12 (IL-12) and gamma interferon, which may activate macrophages to destroy intracellular parasites (Khan et al., 1997; Long et al., 1998; Baszler et al., 1999; Long and Baszler, 2000; Tanaka, Hamada et al., 2000; Tanaka, Nagasawa et al., 2000; Nishikawa, Tragoolpua et al., 2001; Lundén et al., 2002). The production of IgG2a specific for *N. caninum* appears to be associated with development of resistance to primary and secondary *N. caninum* infection (Long et al., 1998; Baszler et al., 1999). Our research group and others have elicited protective immunity against congenital transmission of *N. caninum* in mice by vaccination with either whole tachyzoite extract (Liddell et al., 1999) or specific *N. caninum* antigens (Nishikawa, Inoue et al., 2001; Nishikawa, Xuan et al., 2001). Provoking a type I response and avoiding a type II response during vaccination appears to be critical to the development of immunity to *N. caninum* challenge (Baszler et al., 2000). The purpose of the present study was to test direct immunization of mice with plasmid deoxyribonucleic acid (DNA) coding for 2 different *N. caninum* antigens for their ability to protect against subsequent *N. caninum* tachyzoite challenge infection during pregnancy. One of the antigens, NcGRA7, is associated with tachyzoite-dense granules and elicits a strong immune response during natural and experimental *N. caninum* infections (Lally et al., 1996; Jenkins et al., 1997). NcsHSP33 is a complementary DNA (cDNA) clone from *N. caninum* tachyzoites that shares homology with a conserved family of small heat shock proteins (unpubl.). The precise function of the sHSPs is unknown; however, their expression is developmentally regulated in various organisms, including the closely related coccidian *Toxoplasma gondii* (Bohne et al., 1995).

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MATERIALS AND METHODS

Neospora caninum tachyzoites (NC-1 strain; Dubey et al., 1988) were maintained in human foreskin fibroblast cells and harvested using the procedures described previously (Liddell et al., 1999). Immediately before the mouse challenge injection, the tachyzoites were subjected to trypan blue stain exclusion assay and were found to be 99% viable.

The plasmids used for injections were based on the pCMVi-luc+ eukaryotic expression vector. Fragments containing the entire open reading frame (ORF) of the *N. caninum* antigens NcGRA7 (GenBank U82229) and NcsHSP33 (GenBank AY155364) were polymerase chain reaction (PCR)–amplified from their respective recombinant pBluescript cDNA clones using sense primers at the predicted methionine start codon and antisense primers at the stop codon (NcGRA7 sense 5′-CCGAGAATTCAAAATGGCCCGACAAGC-3′ and antisense 5′-CGCAGGATCCTAACTATTCGGTGTCTAC-3′; NcsHSP33 sense 5′-CCGAGAATTCGCCATGGCGGACTCCGG-3′ and antisense 5′-GCCGGATCCAAATCACTGCACATCAATC-3′). The primers were designed to contain an EcoRI site at the 5′ end of the ORF and a BamHI site at the 3′ end to permit in-frame cloning into the pCMVi-luc+ vector. The amplified, digested fragments were cloned into the pCMVi-luc+ vector, which had been digested with *Eco*RI and *Bam*HI and agarose gel purified to remove the 1.7-kb-long fragment containing the luciferase gene fragment. The resultant recombinant plasmids pCMVi–NcGRA7 and pCMVi–NcsHSP33 were introduced into *Escherichia coli* DH5α cells using standard procedures (Sambrook et al., 1989). The region spanning the cloning sites and the entire ORFs of the *N. caninum* antigen sequences were verified by DNA sequencing on an ABI 373 sequencer. For mouse injections, a control nonrecombinant (NR) vector minus the luciferase sequence (pCMVi-NR) was constructed. Endotoxin-free plasmid DNA was prepared for vaccination using the PerfectPrep Plasmid Maxi Kit according to instructions provided by the manufacturer (Eppendorf, Westbury, New York). The integrity of the plasmids was verified by agarose gel electrophoresis; the major part of each preparation was in supercoiled form. Plasmid DNA concentrations were estimated by measuring absorbance at 260 nm. Plasmid DNA was suspended in 0.15 M NaCl immediately before injection into mice.

Expression of NcGRA7 and NcsHSP33 in vitro

To demonstrate that the *N. caninum* antigens were efficiently expressed from the pCMVi recombinant plasmids, human foreskin fibroblast cells (Hs68, American Type Culture Collection accession no. CRL 1635, Manassas, Virginia) were transfected with pCMVi–NcGRA7, pCMVi–NcsHSP33, or NR vector pCMVi-luc+ or pCMVi using a nonliposomal lipid reagent (Effectene, Qiagen, Valencia, California) according to the manufacturer's guidelines. Six-well tissue culture plates were seeded with fibroblast cells (2×10^5 cells/well) in Dulbecco minimal essential medium (DMEM) containing 500 mg/L glucose, 4 mM L-glutamine, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic

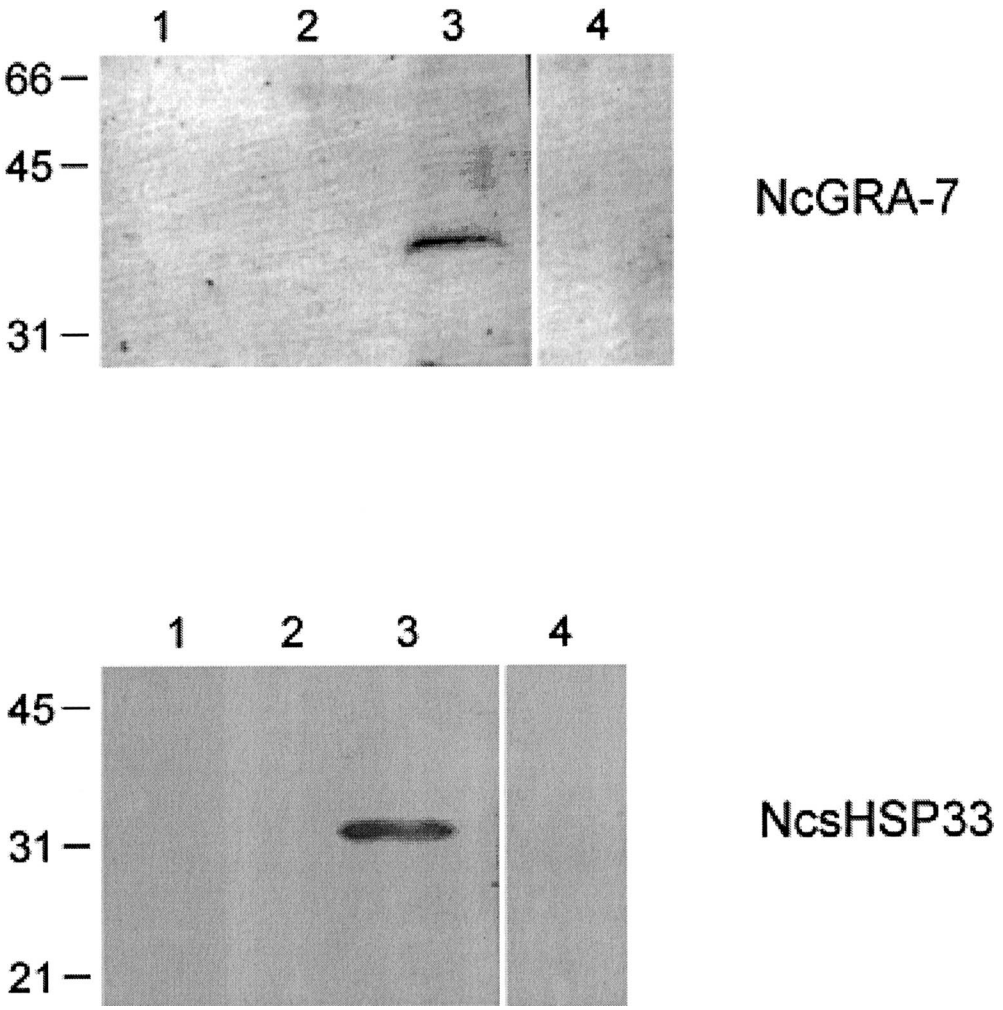

FIGURE 1. Immunoblots of in vitro expressed NcGRA7 and NcsHSP33. Protein extracts of fibroblast cells transfected with pCMVi–NcGRA7 (**A,** lanes 3 and 4), pCMVi–NcsHSP33 (**B,** lanes 3 and 4), or NR vectors pCMVi (**A–B,** lane 1) or pCMVi-luc+ (**A–B,** lane 2). Membrane **A:** lanes 1–3 were probed with anti-NcGRA7 serum and lane 4 with nonimmune serum. Membrane **B:** lanes 1–3 were probed with anti-NcsHSP33 serum and lane 4 with nonimmune serum. Molecular weight markers (kDa) are shown on the left.

acid, 100 U/ml penicillin, and 0.1 mg/ml streptomycin; supplemented with 5% fetal bovine serum; and grown at 37 C in a humidified 5% CO_2 incubator until the cells reached approximately 80% confluency. For each transfection, 0.4 μg DNA was brought up to a volume of 60 μl with DNA condensation Effectene Buffer, mixed with 3.2 μl enhancer, and incubated at room temperature for 5 min. After the addition of 10 μl Effectene Transfection Reagent, the mixture was incubated at room temperature for a further 5–10 min before dilution with 0.4 ml of DMEM media and added to cells that were in 1.6 ml of fresh serum-free medium. The cells were harvested 48 hr after transfection, suspended in sample buffer (Laemmeli, 1970), and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the SDS-PAGE–fractionated proteins were transferred to Immobilon membrane using a semidry blotter (Trans-Blot Semi-Dry Electrophoresis Transfer Cell, BIO-RAD, Hercules, California). The membranes were treated with PBS–2% nonfat dry milk for 1 hr at room temperature to block non–specific antibody binding in subsequent steps. After blocking, individual lanes were probed for 2 hr at 1:1,000 dilution of anti-NcGRA7 or anti-NcsHSP33 serum raised against the respective recombinant proteins in rabbits (Lally et al., 1997), followed by biotinylated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, Missouri). Sera taken from the same rabbits before immunization were used as controls. Binding of rabbit antibody was assessed by treating membranes with 0.5 mg/ml biotinylated goat anti-rabbit Ig (Sigma) followed by 0.25 mg/ml avidin peroxidase (Sigma). Bound antibodies were detected by incubating with 0.5 mg/ml 4-chloro-1-napthol and 0.015% H_2O_2. The strips were washed 3 times between each step with PBS–Tw20.

Vaccinations, mating, and challenge

Adult BALB/c female mice (10 per group, National Cancer Institute, Frederick, Maryland) were injected in the tibialis anterior muscles of both hind limbs with a total dose of 100 μg of pCMVi–NcGRA7, pCMVi–NcsHSP33, or NR pCMVi plasmid DNA in 0.15 M NaCl. Using the same procedure, a booster injection was given 3 wk later. As a positive control for vaccine efficacy, a separate group of female mice was immunized subcutaneously with 5 μg of a crude extract of *N. caninum* tachyzoites using established procedures (Liddell et al., 1999). One week after booster injections, mouse matings were set up (as described by Liddell et al., 1999b). All female mice were challenged on the same day with 1×10^5 *N. caninum* tachyzoites at days 10–12 of gestation. A control group of female mice remained uninfected.

Detection of *N. caninum* DNA in offspring of vaccinated dams

Pups born to experimental or control vaccinated dams were necropsied for brain and lungs at 7 days of age using procedures described previously (Liddell et al., 1999). DNA extracted from these tissues was subjected to the Nc5 *Neospora*-specific PCR assay (Müller et al., 1996; Yamage et al., 1996). Procedures for PCR analysis were as described

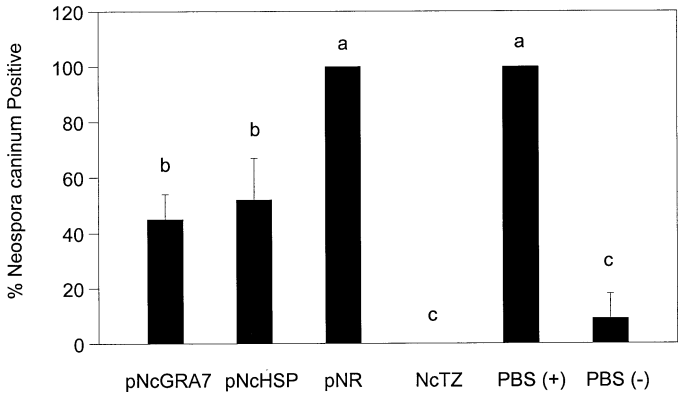

FIGURE 2. Protection against vertical transmission of *Neospora caninum* tachyzoites in pups born from mothers immunized directly with pNcGRA7, pNcsHSP33, or pNR (NR) plasmid DNA. Control groups that were challenged with *N. caninum* tachyzoite included pups born from dams immunized with NcTZ (positive vaccine) or PBS (PBS[+], negative vaccine). PBS(−) controls were not challenged with *N. caninum* tachyzoites. Treatments labeled with different letters are statistically different (alpha < 0.05) by exact binary logistic regression analysis.

previously (Liddell et al., 1999). Amplification reactions were subjected to polyacrylamide gel electrophoresis, ethidium bromide staining, and image capture to a CCD camera. An internal standard of an Nc5 competitor molecule was included in each amplification reaction to control for false-negative PCR (Liddell et al., 1999a). Appropriate positive (*N. caninum* tachyzoite genomic DNA) and negative (H_2O) controls were included in each set of reactions. A minimum of 3 separate, independent PCRs were performed for each sample.

Statistical analysis

Observed counts of *N. caninum*–positive and –negative pups were subjected to exact binary logistic regressions (Hosmer and Lemeshow, 1989) using LogXact4 for Windows (Cytel Software Corporation, Cambride, Massachusetts). In this analysis, the groups receiving NR pCMVi plasmid DNA (pCMViNR), the positive control *N. caninum* tachyzoite (NcTZ) vaccine, PBS followed by *N. caninum* tachyzoite challenge, or PBS with no subsequent challenge were specified as the reference treatment in separate logistic regression analyses. Odds ratios and associated confidence intervals were calculated for each of the treatments with respect to the reference treatment.

RESULTS

Expression of NcGRA7 and NcsHSP33 in vitro

DNA fragments comprising the entire predicted ORF of 2 *N. caninum* antigens NcGRA7 and NcsHSP33 were amplified and cloned in a eukaryotic expression vector (pCMVi). Immunoblotting of fibroblast cells transfected with pCMVi–NcGRA7 or pCMVi–NcsHSP33 showed that the *N. caninum* recombinant proteins were expressed in vitro from this plasmid vector (Fig. 1). Antiserum to recombinant NcGRA7 recognized a protein of approximately 36 kDa in cells transfected with pCMVi–NcGRA7 (Fig. 1A, lane 3), which was not present in cells transfected with plasmid vector controls pCMVi or pCMVi-luc+ (Fig. 1A, lanes 1 and 2). Serum taken before immunization from the same rabbit with recombinant histidine-tagged NcGRA7 did not recognize this protein (Fig. 1A, lane 4). Similarly, anti-NcsHSP33 serum recognized a single band of approximately 31.5 kDa present only in pCMVi-NcsHSP33–transfected cells (Fig. 1B, lane 3), which was not recognized by nonimmune serum (Fig. 1B, lane 2).

TABLE I. Protection against vertical transmission of *Neospora caninum* in pups born to BALB/c mice immunized with plasmid DNA encoding NcGRA7 or NcsHSP33 antigens or with nonrecombinant (NR) plasmid DNA. Data are expressed as the number of pups positive for *N. caninum* DNA by PCR/total number of pups in the litter.

Vaccination	Nc TZ challenge*	No. of positive pups/total no. of pups	
		Vaccine trial 1	Vaccine trial 2
pCMV–NcGRA7	Yes	2/6	3/7
		5/7	2/6
		1/2	
pCMV–NcsHSP33	Yes	2/5	4/6
		6/6	1/6
		3/6	2/5
pCMV–NR	Yes	5/5	2/2
		5/5	4/4
		6/6	7/7
			3/3
			3/3
			5/5
NcTZ†	Yes	0/3	0/5
			0/5
			0/5
			0/3
			0/7
PBS	Yes	1/1	7/7
		3/3	8/8
		2/2	3/3
PBS	No	0/8	0/4
		0/5	1/6‡
		0/7	
		0/2	
		0/7	

* NcTZ challenge, *N. caninum* tachyzoite challenge infection.
† NcTZ, phosphate-buffered saline (PBS)–insoluble whole *N. caninum* tachyzoite antigen.
‡ Contamination error.

Protection of DNA vaccination with NcGRA7

To evaluate the effect of vaccinating with plasmids bearing parasite antigens on transmission of *N. caninum* to offspring, 2 experiments were conducted in a vertical infection mouse model. Immunization of female mice with pCMVi–NcGRA7 followed by challenge with *N. caninum* tachyzoites during gestation elicited partial protection against congenital infection of pups. An average of 46% of pCMVi-NcGRA7–immunized mice were *N. caninum* positive in trials 1 and 2, whereas 100% of pups born from control pCMViNR plasmid–immunized mice were found to contain detectable levels of *N. caninum* DNA in both the trials conducted (Fig. 2; Table I). The reduction in the number of infected offspring in the NcGRA7 group represented a significant decrease ($P < 0.05$; Table I) compared with both pCMVi-immunized (pCMViNR) and nonimmunized challenge infection (PBS+) control groups. Immunization with pCMVi–NcsHSP33 also elicited partial (53% *N. caninum* positive) and significant ($P < 0.05$) level of protection against congenital *N. caninum* infection compared with NR pCMViNR control group or nonimmunized challenge infection (PBS+) offspring (Fig. 2; Table I). The level of protection conferred by immunization with plasmid DNA encoding NcGRA7 or NcsHSP33 was lower

than that observed after immunization with the *N. caninum* tachyzoite vaccine (Fig. 2; Table I).

DISCUSSION

This study showed that immunization of mice with plasmid DNA expressing NcGRA7 or NcsHSP33 antigen elicited partial protection against congenital neosporosis. A significantly ($P < 0.05$) lower percentage of pups were positive for *N. caninum* DNA born to dams immunized with pCMVi–NcGRA7 or pCMVi–NcsHSP33 compared with pups born to pCMViNR-immunized control mice. Although this level of protection was not as high as immunization with tachyzoite extract, no litter from the NcGRA7 and only 1 litter from the NcHSP33 groups had all pups that were PCR positive. This is in contrast to litters from dams immunized with NR plasmid, wherein all pups were positive for *N. caninum*. The ranges of the percentage of *N. caninum*–positive pups from NcGRA7-immunized dams and especially from NcsHSP33-immunized dams were somewhat high. For instance, 51% (range 33–71%) of pups in trial 1 and 38% (range 33–43%) of pups in trial 2 (average = 46%) were born from pCMV-NcGRA7–immunized mice (Table I). Likewise, mice immunized with pCMVi–NcsHSP33 showed partial and somewhat more variable protection against congenital transfer of *N. caninum* to offspring. In these groups, 63% (range 40–100%) of pups in trial 1 and 41% (range 17–67%) of pups in trial 2 (average = 53%) were born from pCMV-NcsHSP33–immunized mice (Table I). These data may reflect some variability in the vaccination or challenge or in the response by individual dams to both. It is interesting that dams immunized with pCMV-NcGRA7 had a heightened antibody response to native NcGRA7, whereas dams immunized with pCMV-HSP33 did not mount a response to native HSP33 (unpubl.). Interpretation of this finding should be treated with caution because type 1 responses and especially the associated cytokines interferon-gamma and IL-12 are believed to play a major role in protective immunity against neosporosis (Khan et al., 1997; Long et al., 1998; Baszler et al., 1999; Long and Baszler, 2000; Tanaka, Hamada et al., 2000; Tanaka, Nagasawa et al., 2000; Nishikawa, Tragoolpua et al., 2001; Lundén et al., 2002).

The level of protection with pNcGRA7 is similar to that achieved in mice using plasmid injection of the GRA7 homologue and surface or rhoptry antigens of *T. gondii* (Nielsen et al., 1999; Desolme et al., 2000; Vercammen et al., 2000; Leyva et al., 2001). Achieving complete protection against congenital neosporosis may require a combination of surface (e.g., SRS2) and internal (e.g., NcGRA7) antigens or coinjection with Th1-stimulating cytokines.

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LITERATURE CITED

BASZLER, T. V., M. T. LONG, T. F. MCELWAIN, AND B. A. MATHISON. 1999. Interferon-gamma and interleukin-12 mediate protection to acute *Neospora caninum* infection in BALB/c mice. International Journal for Parasitology **29:** 1635–1646.

———, T. F. MCELWAIN, AND B. A. MATHISON. 2000. Immunization of BALB/c mice with killed *Neospora caninum* tachyzoite antigen induces a type 2 immune response and exacerbates encephalitis and neurological disease. Clinical and Diagnostic Laboratory Immunology **7:** 893–898.

BOHNE, W., U. GROSS, D. J. FERGUSON, AND J. HEESEMANN. 1995. Cloning and characterization of a bradyzoite-specifically expressed gene (HSP30/BAG1) of *Toxoplasma gondii*, related to genes encoding small heat-shock proteins of plants. Molecular Microbiology **16:** 1221–1230.

DESOLME, B., M. N. MEVELEC, D. BUZONI-GATEL, AND D. BOUT. 2000. Induction of protective immunity against toxoplasmosis in mice by DNA immunization with a plasmid encoding *Toxoplasma gondii* GRA4 gene. Vaccine **18:** 2512–2521.

DUBEY, J. P. 1999. Neosporosis in cattle: Biology and economic impact. Journal of the American Veterinary Medical Association **214:** 1160–1163.

———, A. L. HATTEL, D. S. LINDSAY, AND M. J. TOPPER. 1988. Neonatal *Neospora caninum* infection in dogs: Isolation of the causative agent and experimental transmission. Journal of the American Veterinary Medical Association **193:** 1259–1263.

HOSMER, D. W., AND S. LEMESHOW. 1989. Applied logistic regression. John Wiley & Sons, New York, 307 p.

JENKINS, M. C., W. WOUDA, AND J. P. DUBEY. 1997. Serological response over time to recombinant *Neospora caninum* antigens in cattle after a neosporosis-induced abortion. Clinical and Diagnostic Laboratory Immunology **4:** 270–274.

KHAN, I. A., J. D. SCHWARTZMAN, S. FONSEKA, AND L. H. KASPER. 1997. *Neospora caninum*: Role for immune cytokines in host immunity. Experimental Parasitology **85:** 24–34.

LAEMMELI, U. K. 1970. Cleavage of structural proteins during assembly of the head of the bacteriophage T4. Nature **227:** 680–685.

LALLY, N., M. JENKINS, AND J. P. DUBEY. 1996. Evaluation of two *Neospora caninum* antigens for use in an enzyme-linked immunosorbent assay for the diagnosis of bovine neosporosis. Clinical and Diagnostic Laboratory Immunology **3:** 275–279.

———, ———, S. LIDDELL, AND J. P. DUBEY. 1997. A dense granule protein (NCDG1) gene from *Neospora caninum*. Molecular and Biochemical Parasitology **87:** 239–243.

LEYVA, R., P. HERION, AND R. SAAVEDRA. 2001. Genetic immunization with plasmid DNA coding for the ROP2 protein of *Toxoplasma gondii*. Parasitology Research **87:** 70–79.

LIDDELL, S., M. C. JENKINS, C. M. COLLICA, AND J. P. DUBEY. 1999. Prevention of vertical transfer of *Neospora caninum* in BALB/c mice by vaccination. Journal of Parasitology **85:** 1072–1075.

———, ———, AND J. P. DUBEY. 1999a. A competitive PCR assay for quantitative detection of *Neospora caninum*. International Journal for Parasitology **29:** 1583–1587.

———, ———, AND ———. 1999b. Vertical transmission of *Neospora caninum* in BALB/c mice determined by polymerase chain reaction detection. Journal of Parasitology **85:** 550–555.

LONG, M. T., AND T. V. BASZLER. 2000. Neutralization of maternal IL-4 modulates congenital protozoal transmission: Comparison of innate versus acquired immune responses. Journal of Immunology **164:** 4768–4774.

———, ———, AND B. A. MATHISON. 1998. Comparison of intracerebral parasite load, lesion development, and systemic cytokines in mouse strains infected with *Neospora caninum*. Journal of Parasitology **84:** 316–320.

LUNDÉN, A., S. WRIGHT, J. E. ALLEN, AND D. BUXTON. 2002. Immunisation of mice against neosporosis. International Journal for Parasitology **32:** 867–876.

MÜLLER, N., V. ZIMMERMANN, B. HENTRICH, AND B. GOTTSTEIN. 1996. Diagnosis of *Neospora caninum* and *Toxoplasma gondii* infection by PCR and DNA hybridization immunoassay. Journal of Clinical Microbiology **34:** 2850–2852.

NIELSEN, H., S. L. LAUEMOLLER, L. CHRISTIANSEN, S. BUUS, A. FOMSGAARD, AND E. PETERSEN. 1999. Complete protection against lethal *Toxoplasma gondii* infection in mice immunized with a plasmid encoding the SAG1 gene. Infection and Immunity **67:** 6358–6363.

NISHIKAWA, Y., N. INOUE, X. XUAN, H. NAGASAWA, I. IGARASHI, K. FUJISAKI, H. OTSUKA, AND T. MIKAMI. 2001. Protective efficacy of vaccination by recombinant vaccinia virus against *Neospora caninum* infection. Vaccine **19:** 1381–1390.

———, K. TRAGOOLPUA, N. INOUE, L. MAKALA, H. NAGASAWA, H. OTSUKA, AND T. MIKAMI. 2001. In the absence of endogenous gamma interferon, mice acutely infected with *Neospora caninum* succumb to a lethal immune response characterized by inactivation of peritoneal macrophages. Clinical and Diagnostic Laboratory Immunology **8:** 811–816.

———, X. XUAN, H. NAGASAWA, I. IGARASHI, K. FUJISAKI, H. OTSUKA, AND T. MIKAMI. 2001. Prevention of vertical transmission of *Neospora caninum* in BALB/c mice by recombinant vaccinia virus carrying NcSRS2 gene. Vaccine **19:** 1710–1716.

SAMBROOK, J., E. F. FRITSCH, AND T. MANIATIS. 1989. Molecular cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 574 p.

TANAKA, T., T. HAMADA, N. INOUE, H. NAGASAWA, K. FUJISAKI, N. SUZUKI, AND T. MIKAMI. 2000. The role of CD4(+) or CD8(+) T cells in the protective immune response of BALB/c mice to *Neospora caninum* infection. Veterinary Parasitology **90:** 183–191.

———, H. NAGASAWA, K. FUJISAKI, N. SUZUKI, AND T. MIKAMI. 2000. Growth-inhibitory effects of interferon-gamma on *Neospora caninum* in murine macrophages by a nitric oxide mechanism. Parasitology Research **86:** 768–771.

VERCAMMEN, M., T. SCORZA, K. HUYGEN, J. DE BRAEKELEER, R. DIET, D. JACOBS, E. SAMAN, AND H. VERSCHUEREN. 2000. DNA vaccination with genes encoding *Toxoplasma gondii* antigens GRA1, GRA7, and ROP2 induces partially protective immunity against lethal challenge in mice. Infection and Immunity **68:** 38–45.

YAMAGE, M., O. FLECHTNER, AND B. GOTTSTEIN. 1996. *Neospora caninum*: Specific oligonucleotide primers for the detection of brain ''cyst'' DNA of experimentally infected nude mice by the polymerase chain reaction (PCR). Journal of Parasitology **82:** 272–279.